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PITX2 and β-Catenin Interactions Regulate Lef-1 Isoform Expression▽

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Lef-1 and PITX2 function in the Wnt signaling pathway by recruiting and interacting with β-catenin to activate target genes. Chromatin immunoprecipitation (ChIP) assays identified the *Lef-1* promoter as a PITX2 downstream target. Transgenic mice expressing *LacZ* driven by the 2.5-kb *LEF-1* promoter demonstrated expression in the tooth epithelium correlated with endogenous *Lef-1 FL* epithelial expression. PITX2 isoforms regulate the *LEF-1* promoter, and β-catenin synergistically enhanced activation of the *LEF-1* promoter in combination with PITX2 and Lef-1 isoforms. PITX2 enhances endogenous expression of the full-length β-catenin-dependent Lef-1 isoform (Lef-1 FL) while decreasing expression of the N-terminally truncated β-catenin-independent isoform. Our research revealed a novel interaction between PITX2, Lef-1, and β-catenin in which the Lef-1 β-catenin binding domain is dispensable for its interaction with PITX2. PITX2 interacts with two sites within the Lef-1 protein. Furthermore, β-catenin interacts with the PITX2 homeodomain and Lef-1 interacts with the PITX2 C-terminal tail. Lef-1 and β-catenin interact simultaneously and independently with PITX2 through two different sites to regulate PITX2 transcriptional activity. These data support a role for PITX2 in cell proliferation, migration, and cell division through differential Lef-1 isoform expression and interactions with Lef-1 and β-catenin.

Pitx2 and *Lef-1* encode two transcription factors whose expression can be regulated by early signaling events involved in numerous developmental programs. *Pitx2* and *Lef-1* are differentially expressed in many tissues, and they demonstrate overlapping expression during tooth development. *Lef-1* can be activated by BMP, Wnt, Smads, and transforming growth factor β signaling (18, 32, 33). Furthermore, Lef-1 transcriptional activity is regulated by its interaction with β-catenin. Secreted Wnt proteins initiate a signaling cascade that prevents degradation of β-catenin in the cytoplasm, and β-catenin subsequently translocates to the nucleus, where it activates Wnt-responsive genes (42). The full-length Lef-1 (Lef-1 FL) isoform, containing the β-catenin binding domain (βBD) has been termed growth promoting because it is expressed during cell growth in undifferentiated, mitotically active cells. This Lef-1 activity is countered by the expression of a truncated Lef-1 protein that lacks the βBD and can compete for binding to Wnt target genes. This shorter Lef-1 transcript (Lef-1 ΔN113) could act as an inhibitory isoform, due to its ability to interact with cofactors that also bind Lef-1 FL, as well as compete for DNA binding sites, and thus has been termed growth suppressing (27, 30, 34). However, both Lef-1 isoforms can stimulate cell differentiation. Interestingly, in colon cancer cells, only the *LEF-1 FL* transcript is produced, and the loss of balance between the long and short forms may affect cancer progression (30, 34). Therefore, it is essential to understand the molecular functions of these two Lef-1 isoforms.

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Tooth development is initiated with a thickening of the ectoderm-derived oral epithelium between embryonic day 10 (E10) and E11. The epithelium undergoes invagination into the neural crest-derived mesenchyme to form a tooth "bud." The bud forms at E12, and at E13, the enamel knot forms at the base of the tooth bud and comprises a signaling center driving tooth morphogenesis. A role for Lef-1 in the development of teeth, whiskers, hair follicles, and mammary glands was identified through targeted inactivation of the *Lef-1* gene in mice (43). In *Lef-1* mutant mice, tooth development is arrested at later stages, and the mice lack teeth. Consistent with this phenotype, *Lef-1* expression can be seen at E10.5 in the dental epithelium, and at E14.5 during cap stage development, *Lef-1* was found in both the dental epithelium and dental papilla mesenchyme (40, 43). From E10 to E12, *Lef-1* transcripts are detected initially in the epithelium and subsequently in the mesenchyme, consistent with the change in the developmental dominance of these tissues (34). The role of *Lef-1* in the mesenchyme is unclear, since an essential function for *Lef-1* expression could be demonstrated only in the dental epithelium between E13 and E14, corresponding to the presence of *Lef-1* transcripts in the epithelial tooth bud (34).

Pitx2 is the earliest transcription marker observed in tooth development and is specifically restricted to the developing dental epithelium (24, 39). In tooth formation, as in all organs, developmental programs are usually initiated by more than one gene and cell type acting in concert to promote cell proliferation, migration, and/or differentiation. Tooth development is arrested in *Pitx2*$^{-/-}$ mice (19, 35, 37). β-*catenin* is expressed at the same time as *Lef-1* and *Pitx2* in the developing tooth bud and epithelial tissues (17).

In order to understand the roles of PITX2, β-catenin, and Lef-1 in tooth and other developmental pathways, we analyzed

transgenic mice expressing *LacZ* under the control of the *LEF-1* promoter. This construct demonstrated expression in the dental epithelium at an early stage (E12.5) of tooth development. The expression of *LEF-1* directly overlaps *Pitx2* and occurs at approximately E1.5 after *Pitx2* expression. Thus, the human *LEF-1* promoter can recapitulate endogenous *Lef-1* expression in the dental epithelium. PITX2 induces *Lef-1 FL* expression in CHO cells, which do not endogenously express this *Lef-1* isoform. Our data reveal β-catenin-dependent and -independent Lef-1 transcriptional activities and interactions with PITX2 to regulate *Lef-1* expression. Lef-1 interactions with PITX2 provide a feedback mechanism that allows the continued expression of *Lef-1*. Changes in *Lef-1* expression to the Lef-1 FL β-catenin-dependent isoform correlates with its activity in cell division, migration, and morphogenesis. *Pitx2* expression is linked to cell division, migration, and morphogenesis, and the coordinated expression of these factors is required for normal embryonic development and provides a new mechanism for the control of these events.

MATERIALS AND METHODS

ChIP analysis. Chromatin immunoprecipitation (ChIP) analysis was performed as described using the ChIP assay kit (Upstate) and as previously described (11). Two primers for amplifying the PITX2 binding site in the *Lef-1* proximal promoter were as follows: sense, starting at −1049 (5′-AGACAGTG GGAACCGGAGAA-3′), and antisense, starting at −668 (5′-ACTCTACTAA CCGTGAATCACAC-3′). Two primers for amplifying the PITX2 binding site in the *Lef-1* distal promoter were as follows: sense, starting at −5657 (5′-GGAG GAGGGCATCAGATCTTATTGTGAGTGGTTG-3′), and antisense, starting at −5245 (5′-TTAGGAGGCAAGGTACATATTTAAGGGGGAGAG-3′). All the PCR products were evaluated on a 2% agarose gel in 1× Tris-borate-EDTA for appropriate size and confirmed by sequencing. As controls, the Lef-1 primers were used without chromatin, normal rabbit immunoglobulin G (IgG) was used to replace the PITX2 antibody to reveal nonspecific immunoprecipitation (IP) of the chromatin, and primers for an unrelated gene were used to demonstrate the specificity of the PITX2 antibody-immunoprecipitated chromatin.

Lef-1 promoter-reporter constructs and the generation of transgenic mice. As previously described, the *LEF-1* expression construct (LF-2700/-200) containing the human *LEF-1* promoter fragment upstream of the β-galactosidase gene (18) was used to generate previously characterized transgenic mice (18). LF-2700/-200 contains sequences in the promoter that span bp −2700 to −200 (relative to the ATG start methionine at +1). Transgenic mice were genotyped by PCR and/or Southern blotting using DNA prepared from tail biopsy specimens. Primer sets EL905 (5′CAAACTTCAGCTTCCCTTCTGCTG) and EL906 (5′ GACGAGG AAGAAGGAACTGAAGAC) were derived from a β-galactosidase transgene and used for PCR screening. These primers identified a 379-bp β-galactosidase transgene band in positive transgenic mice.

Histochemical detection of β-galactosidase activity. Timed pregnancies were established between C57BL/6J males and heterozygous, transgene-positive females. The appearance of a vaginal plug on the morning following breeding was designated as E0.5. PCR of DNA harvested from the yolk sac or part of the embryo determined the genotypes of the embryos. Embryos and/or tissue samples were dissected in cold phosphate-buffered saline (PBS) at designated ages and fixed (0.2% glutaraldehyde, 5 mM EGTA, pH 7.3, 2 mM $MgCl_2$, 0.02% NP-40, 0.01% deoxycholate, 2% paraformaldehyde, 0.1 M sodium phosphate, pH 8.8) for 15 to 60 min at room temperature with rocking. After being washed in detergent solution (0.02% NP-40, 0.01% deoxycholate, 2 mM $MgCl_2$ in 0.1 M sodium phosphate, pH 8.0) three times for 10 min each time at room temperature, the embryos were stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) solution (1 mg/ml X-Gal, 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide in the wash buffer) in the dark at 37°C for 3 h. They were then incubated at room temperature overnight. The stained embryos were postfixed in 0.5% glutaraldehyde-10% formalin after being washed three times in PBS. The embryos were equilibrated in 80% glycerol-PBS (containing 0.5% Triton X-100) prior to being photographed. When histologic sections were evaluated, the embryos were equilibrated in 30% sucrose and embedded in optimal-cutting-temperature compound. Sections were counterstained with propidium iodide prior to being photographed.

In situ hybridization. The mouse PITX2C full-length cDNA and the proline-rich region (450 bp) of the mouse Lef-1 cDNA (GenBank accession no. 6754531) were cloned into a pBlueScript KS plasmid (Stratagene, La Jolla, CA) using KpnI and EcoRV restriction sites. This region does not overlap with conserved high-mobility group (HMG) DNA binding domains. The sense and antisense digoxigenin-labeled RNA probes were prepared by in vitro transcription using a digoxigenin-RNA labeling kit with T3 and T7 RNA polymerase in accordance with the manufacturer's instructions (Roche Molecular Systems, Pleasanton, CA). Whole-mount hybridization was performed essentially as previously described (25, 44); however, a higher temperature (70°C) was employed during the hybridization and washing steps. In situ hybridizations on cryosections were performed as previously described (13–15) with the above-mentioned digoxigenin-labeled RNA probes.

Immunohistochemistry. Mouse embryos were treated with 4% paraformaldehyde and dehydrated with sequential concentrations of alcohol and finally with xylene. Then, the embryos were embedded in paraffin, and sections were made at 5-μm thickness. The sections were deparaffinized and treated with 0.1 M sodium citrate buffer for 7.5 min at 100% power in a revolving 800-W microwave with an additional three cycles of 5 min at 50% power. Subsequently, the sections were treated with 4% hydrogen peroxide to block the endogenous peroxidase activity. Then, the slides were incubated with 5% goat serum for 30 min, followed by overnight incubation with anti-β-catenin antibody at 1/400 dilution (Chemicon International). The following day, the slides were treated with biotinylated anti-rabbit secondary antibody (Vector Laboratories) at a concentration of 1/200 for 30 min. Avidin biotin complex and DAB substrate (Vector Laboratories) were used as our reporting system. Negative controls were treated exactly the same except for the primary antibody; PBS with 5% blocking goat serum was used.

Expression and reporter constructs. Expression plasmids containing the cytomegalovirus (CMV) promoter linked to the *PITX2* cDNA were constructed in pcDNA 3.1 MycHisC (Invitrogen) (1, 2, 9). Lef-1 and β-catenin S37A expression plasmids have been previously described (18). Lef-1 deletion clones were made using nested primers and cloned into the pcDNA 3.1 MycHisC vector. The Lef-1 deletion constructs were prepared by PCR amplification of the full-length *Lef-1* cDNA and cloned into the pcDNA3.1 MycHisC vector. The human *LEF-1* promoters have been previously described and were PCR amplified and cloned into the luciferase vector as previously described (41). All constructs were confirmed by DNA sequencing. A simian virus 40 (SV-40) or CMV β-galactosidase reporter plasmid was cotransfected in all experiments as a control for transfection efficiency. All plasmids were double-banded CsCl purified.

Western blot assays. Expression of endogenous or transiently expressed PITX2, Lef-1, and β-catenin proteins was demonstrated using the PITX2 P2R10 antibody (24) and Lef-1 and β-catenin antibodies (Upstate). The Lef-1 mouse monoclonal antibody from Upstate recognizes the 57- to 55-kDa proteins, as well as the short isoform. Approximately 10 to 40 μg of transfected cell lysates were analyzed in Western blots. Following SDS gel electrophoresis, the proteins were transferred to polyvinylidene difluoride (PVDF) filters (Millipore), immunoblotted, and detected using specific antibodies and enhanced-chemiluminescence (ECL) reagents from GE HealthCare.

Cell culture, transient transfections, and luciferase and β-galactosidase assays. CHO, C3H10T1/2, and LS-8 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% or 10% fetal bovine serum and penicillin/streptomycin and transfected by electroporation. The cultures were fed 24 h prior to transfection, resuspended in PBS, and mixed with 2.5 μg of expression plasmids, 5 μg of reporter plasmid, and 0.5 μg of CMV or SV-40 β-galactosidase plasmid. Electroporation of CHO cells was performed at 360 V and 950 μF (Gene Pulser XL; Bio-Rad) and that of C3H10T1/2 cells at 380 V and 950 μF. LS-8 cells were transfected by electroporation as previously described (22). The transfected cells were incubated for 24 h in 60-mm culture dishes and fed with 5% fetal bovine serum and Dulbecco's modified Eagle's medium and then lysed and assayed for reporter activities and protein content by Bradford assay (Bio-Rad). Luciferase was measured using reagents from Promega. β-Galactosidase was measured using the Galacto-Light Plus reagents (Tropix Inc.). All luciferase activities were normalized to β-galactosidase activity.

Expression and purification of glutathione *S*-transferase (GST) fusion proteins. The human *PITX2A* and *-C* constructs were PCR amplified from cDNA clones as described previously (1, 2). The *PITX2*, β-*catenin*, and *Lef-1* PCR products were cloned into the pGex6P2 GST vector (Amersham Pharmacia Biotech) as previously described (1, 2). All pGex6P-2 GST plasmids were confirmed by DNA sequencing. The plasmids were transformed into BL21 cells. Proteins were isolated as described previously (2, 9). All proteins were cleaved from the GST moiety with PreScission protease (GE HealthCare). Protein concentrations were quantitated with Bradford Reagent (Bio-Rad Laboratories, Hercules, CA). Proteins were examined by electrophoresis on denaturing SDS-

polyacrylamide gels, followed by Coomassie blue staining (50% methanol, 10% acetic acid, and 0.5% Coomassie brilliant blue stain).

GST-PITX2 pull-down assays. Immobilized GST–Lef-1 and GST-PITX2 fusion proteins were prepared as described above and suspended in binding buffer (20 mM HEPES, pH 7.5, 5% glycerol, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol with or without 1% milk and 400 μg/ml of ethidium bromide). Purified bacterially expressed PITX2, β-catenin, or Lef-1 protein (80 to 100 ng) was added to 5 μg immobilized GST–Lef-1, GST-PITX2, or truncated fusion proteins or GST, respectively, in a total volume of 100 μl and incubated for 30 min at 4°C. The beads were pelleted and washed four times with 200 μl binding buffer. The bound proteins were eluted by boiling them in SDS sample buffer and separated on either 10% or 12.5% SDS-polyacrylamide gels or 15% tricine gels. The experiment to analyze cobinding of β-catenin and Lef-1 ΔN113 to PITX2 was performed by adding purified β-catenin and Lef-1 ΔN113 to the appropriate GST-PITX2 constructs. After incubation and extensive washing, the beads were divided into aliquots, run on an SDS gel, and probed with either β-catenin or Lef-1 antibodies. Thus, both Lef-1 ΔN113 and β-catenin were present in the binding reactions. Purified PITX2C, β-catenin, or Lef-1 protein was analyzed in a Western blot. Following SDS gel electrophoresis, the proteins were transferred to PVDF filters (Millipore), immunoblotted, and detected using the PITX2, β-catenin, or Lef-1 antibody (Upstate) and ECL reagents from GE HealthCare.

RT-PCR. CHO cells were transfected with empty expression vector (mock) or pCMV-PITX2C, and cells were harvested 48 h after transfection. Total RNA was isolated as previously described (41). Reverse transcription was preformed using 5 μg of total RNA, random primers, iScript reverse transcriptase (RT), and the iScript Select cDNA synthesis kit (Bio-Rad) in a total volume of 20 μl. The reaction mixture was incubated according to the manufacturer's directions. The cDNA (2 μl) was used in the PCR with Advantage polymerase (Clontech) and the appropriate primers. The sense primer for amplification of the Lef-1 FL isoform was 5′-GCAGCGGAGCGGAGATTACACAG-3′, and the antisense primer was 5′-TCCCTTGTTGTACAGGCCTCCGTC-3′. The primer pair for the Lef-1 short isoform was 5′-CGGCGTTGGACAGATCAC-3′ (sense) and 5′-TTGATAGCTGCACTCTCC-3′ (antisense). Products were analyzed on an agarose gel, and bands were isolated and sequenced to confirm their identities.

IP assay. Approximately 24 h after cell transfection with PITX2 and Lef-1 FL, CHO cells were rinsed with 1 ml of PBS and then incubated with 1 ml ice-cold RIPA buffer for 15 min at 4°C. Cells were harvested and disrupted by repeated aspiration through a 25-gauge needle attached to a 1-ml syringe. The lysates were then incubated on ice for 30 min. Cellular debris was pelleted by centrifugation at 10,000 × *g* for 10 min at 4°C. An aliquot of lysate was saved for analysis as an input control. The supernatant was transferred to a fresh 1.5-ml microcentrifuge tube on ice and precleared using the mouse ExactaCruz C IP matrix (Santa Cruz Biotechnology) for 30 m at 4°C. The matrix was removed by brief centrifugation, and the supernatant was transferred to a new tube. An IP antibody-IP matrix complex was prepared according to the manufacturer's instructions, using primary anti-Lef-1 (1.7 μl) antibody (Upstate). The IP antibody-IP matrix complex was incubated with the precleared cell lysate at 4°C for 12 h. After incubation, the lysate was centrifuged to pellet the IP matrix. The matrix was washed two times with PBS and resuspended in 15 μl of double-distilled H_2O and 3 μl 6× SDS loading dye. Samples were boiled for 5 m and resolved on a 10% polyacrylamide gel. Western blotting was used with anti-PITX2 antibody and horseradish peroxidase-conjugated rabbit ExactaCruz C reagent to detect immunoprecipitated proteins.

RESULTS

Pitx2 binds to the *Lef-1* promoter in vivo. The mouse *Lef-1* promoter contains multiple Pitx2 binding sites (bicoid sites) (Fig. 1A). ChIP analyses were done in LS-8 oral/dental epithelial cells to demonstrate Pitx2 binding to the *Lef-1* promoter because the LS-8 cell line endogenously expresses Pitx2 and Lef-1 (11, 22). A primer pair flanking the Pitx2 binding site located at −779 to −784 in the proximal *Lef-1* promoter produced a 381-bp product (Fig. 1A). Chromatin isolated from cells prior to IP served as an input control. The primer set amplified the *Lef-1* promoter from chromatin input derived from the LS-8 cell line (Fig. 1B, lane 4). A PCR was performed without chromatin and with primers only as a negative control (Fig. 1B, lane 3). Endogenous Pitx2 was bound to the *Lef-1* promoter in vivo, as shown using PITX2 antibody and Lef-1 primers (Fig. 1B, lane 2). Normal rabbit IgG was used as a control and did not immunoprecipitate the *Lef-1* promoter (Fig. 1B, lane 5). Furthermore, primers for an unrelated gene did not amplify a product from the PITX2 antibody-immunoprecipitated chromatin (Fig. 1B, lane 6). A second ChIP assay was performed to demonstrate Pitx2 binding to a distal element in the *Lef-1* promoter. A primer pair flanking the Pitx2 binding site located at −5447 to −5452 in the distal *Lef-1* promoter produced a 412-bp product (Fig. 1A). The assay was performed as shown in Fig. 1B using different primers and with the addition of the control primer PCR product from the chromatin input to demonstrate that the control primers produced the correct product (Fig. 1C). All PCR products were sequenced to confirm their identities.

The transcriptional activity of the 2.5-kb human *LEF-1* promoter is spatially restricted to the early bud stage and vestibular lamina of developing teeth. During tooth morphogenesis, *LacZ* expression was absent at the epithelial-thickening stage of tooth formation in E11.5 embryos (Fig. 2A). However, transgene expression was seen where the incisor and molar teeth developed at E12.5 (Fig. 2B). Transgene expression during molar tooth development was observed only in E12.5 embryos, in which *Lef-1* gene regulation has also been implicated (32). However, molar expression of the *LEF-1* promoter disappeared in E13.5 embryos (Fig. 2C). Conversely, *LEF-1* promoter expression during incisor tooth development persisted from E12.5 to E17.5 (Fig. 2B, C, E, and F), and higher magnification revealed robust LacZ staining in the incisors (Fig. 2C, E, and F, insets). Transgene-negative littermates did not express LacZ at any developmental time points (Fig. 2D and data not shown). The transgenic reporter was also expressed in the whisker follicles, demonstrating that the *LEF-1* promoter contains elements necessary for specific expression at sites known for endogenous *Lef-1* expression (Fig. 2C and E). Sections of mouse incisor tooth buds at E13.5 and E14.5 demonstrated robust LacZ expression throughout the dental epithelium of the tooth bud and developing cap stage and the dental lamina (Fig. 2G and H).

Although expression of the 2.5-kb *LEF-1* promoter overlapped with endogenous *Lef-1* mRNA (Fig. 2I and J), no transgene expression was detected in the mesenchyme during the cap stage. This reflects a divergence from where the Lef-1 mRNA and protein are normally seen. These findings suggest that the 2.5-kb *LEF-1* promoter contains sufficient information to regulate expression properly during the initial bud-forming stage of tooth development and during the stages of vestibular-lamina formation. Interestingly, this region of the promoter was expressed only in epithelium-derived cell types of the dental organ. *Pitx2* expression is also restricted to the dental epithelium and is not observed in the mesenchyme-derived cell types (23). Expression restricted to only certain epithelial cell types of the dental organ also suggests that diverse transcriptional programs regulate different regions of the *LEF-1* promoter during tooth development. β-Catenin was expressed in the epithelium and condensing mesenchyme in tooth incisors at E14.5 and was found to be both nuclear and cytoplasmic (Fig. 2K to M).

***Pitx2* and *Lef-1* are coexpressed in the developing mouse incisor.** *Pitx2* expression was detected in E12.5 incisors, while

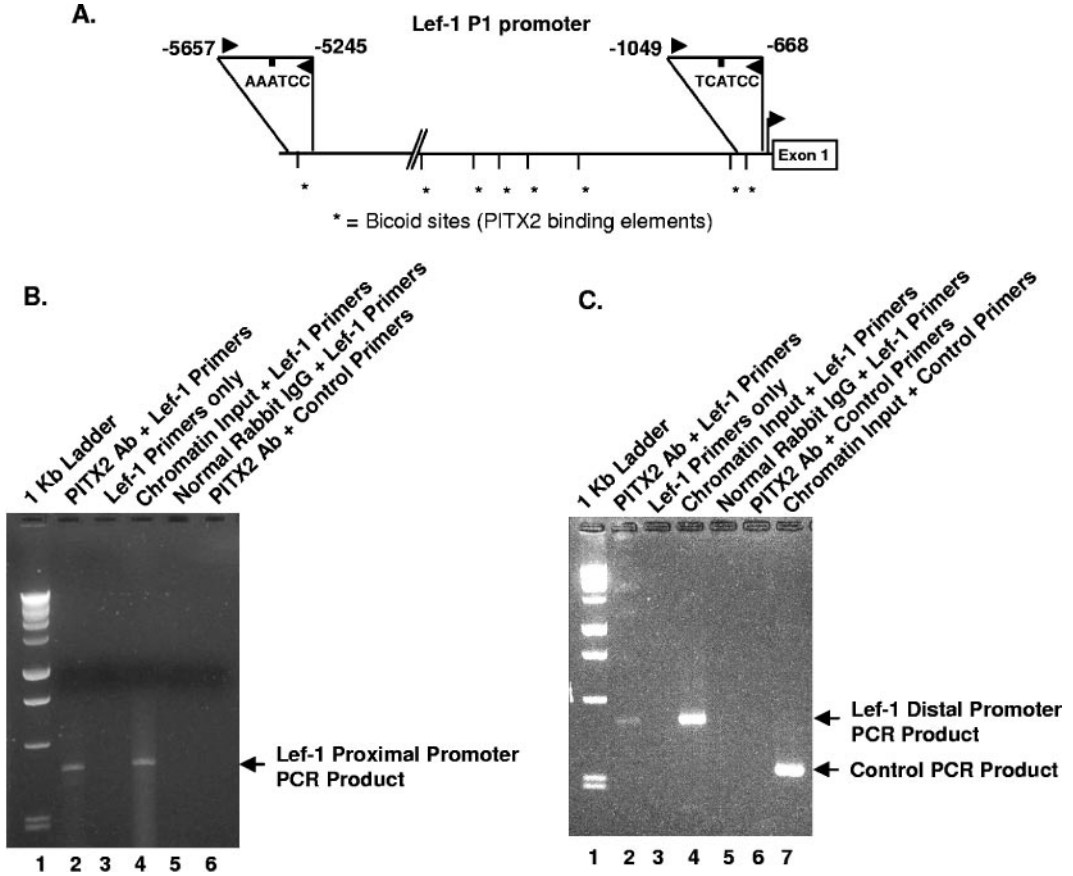

FIG. 1. Pitx2 binds to the *Lef-1* promoter in vivo. (A) Schematic of the mouse *Lef-1* promoter, with the Pitx2 binding sites and locations of the sense primers and the antisense primers indicated. (B) ChIP assays of the *Lef-1* proximal promoter were performed using LS-8 cells. Lane 1 contains the 1-kb ladder, and lane 2 is the Pitx2 antibody (Ab)-immunoprecipitated chromatin amplified using the specific *Lef-1* promoter primers that produced the correct-size product of 381 bp. Lane 3 is Lef-1 primers only; lane 4 is the chromatin input using the *Lef-1* promoter primers. Lane 5 is the IP using normal rabbit IgG and Lef-1 primers, and lane 6 is the PITX2 Ab-immunoprecipitated chromatin amplified with primers for an unrelated gene. (C) ChIP assays of the *Lef-1* distal promoter were performed using LS-8 cells. All lanes are as in panel B, except for different primer sets and lane 7 containing the PCR product with the control primers and chromatin input.

comparatively low levels of *Lef-1* expression were observed at the same time point (compare Fig. 3C to D). *Pitx2* expression increased by E14.5 in the incisors (Fig. 3E). Furthermore, *LacZ* expression from the *LEF-1* promoter in the transgenic mouse was observed at E14.5, demonstrating specific expression of the human *LEF-1* promoter in the mouse incisors (Fig. 3F). As controls, sense probes were used for *Pitx2* and *Lef-1* expression in E12.5 whole-mount embryos (Fig. 3A and B) and did not give a signal.

PITX2 activates endogenous expression of the Lef-1 FL isoform in CHO cells. A tooth epithelial cell line, LS-8; a pluripotent cell line, C3H10T1/2; and CHO cell lysates were assayed for endogenous Lef-1 protein by Western blotting. We previously showed that the LS-8 and C3H10T1/2 cells endogenously express the 57-, 55-, and 39-kDa Lef-1 isoforms (11). The Lef-1 FL isoform is produced from the *Lef-1* P1 promoter, and the N-terminally truncated Lef-1 ΔN113 isoform is synthesized from the Lef-1 P2 promoter in intron 2 (Fig. 4A) (30, 34). The 55- and 57-kDa Lef-1 products are produced from the same P1 promoter and may arise from alternative splicing or posttranslational modifications (30, 34, 45). However, CHO cells endogenously express only the 39-kDa isoform (Fig. 4B). PITX2 induced expression of the 57-kDa Lef-1 FL isoform in CHO cells, which do not endogenously express *Pitx2* (Fig. 4B). Interestingly, an ~60-kDa band recognized by the Lef-1 antibody was observed in the CHO cell lysates (Fig. 4B). This band disappeared after PITX2 expression in CHO cells (Fig. 4B). The nature of this band is unknown, and it has not been previously reported. To confirm the expression of the 57-kDa band in the Western blot, an RT-PCR assay was performed using RNA isolated from PITX2-transfected CHO cells. The short Lef-1 transcript was detected in both mock- and PITX2-transfected cells (Fig. 4C, lanes 3 and 4). However, primers that amplify the Lef-1 FL isoform did not produce a PCR product from the mock-transfected cells but did detect the Lef-1 FL transcript from the PITX2-transfected cells (Fig. 4C, lanes 6 and 7). As a control, β-actin primers detected equal amounts of β-actin transcripts from both mock- and PITX2-transfected cells (Fig. 4B, lanes 9 and 10). All RT-PCR products were sequenced to confirm their identities. β-Catenin is highly expressed in both LS-8 and 10T1/2 cells, but relatively low expression was observed in CHO cells (Fig. 4D). These data suggest that cells expressing Pitx2 regulate endogenous Lef-1 isoform expression.

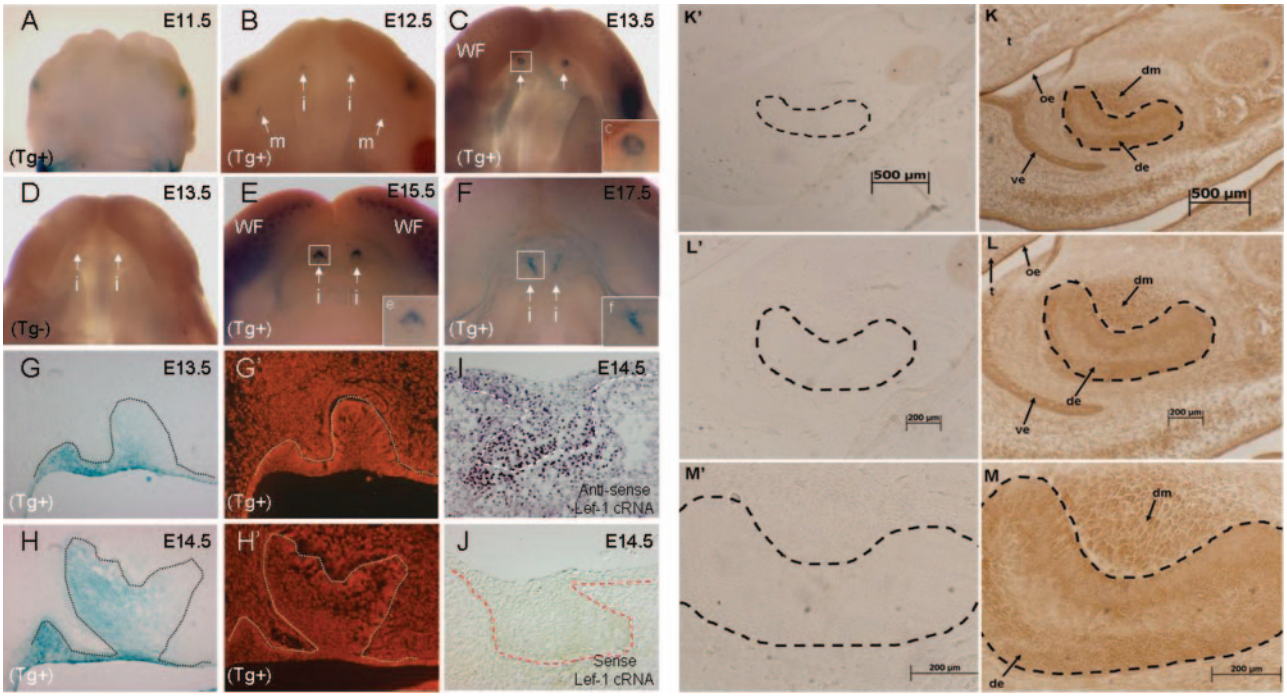

FIG. 2. Expression of the LF-2700 transgenic *LEF-1* promoter during tooth morphogenesis. (A to F) Whole-mount X-Gal staining of transgenic (Tg+) (A to C, E, and F) and nontransgenic (Tg−) (D) littermates demonstrated LacZ transgene expression in incisors (i) and molars (m) for the indicated developmental time points. The insets (c, e, and f) show enlargements of the boxed regions in the same panels of whole-mount staining. (G and H) Parasagittal sections of whiskers of E13.5 and E14.5 embryos indicated the expression of LEF-1–LacZ in the dental lamina and epithelium but not the mesenchyme of the tooth. (G′ and H′) Propidium iodide staining of the sections corresponding to panels G (G′) and H (H′). (I) In situ hybridization using a Lef-1–cRNA probe demonstrated *Lef-1* expression in the dental epithelium and mesenchyme of an incisor at the initiation of the cap stage (E14.5). (J) No signal was detected using a Lef-1 sense probe. (K, L, and M) Sagittal sections and immunohistochemical assays of E14.5 mouse incisors using a β-catenin antibody and DAB staining at ×4, ×10, and ×20 magnifications, respectively. K′, L′, and M′) Control (secondary antibody only) sections corresponding to panels K, L, and M. The sections are not counterstained to reveal all areas of β-catenin staining. The incisor tooth bud is outlined. WF, whisker follicle; de, dental epithelium; oe, oral epithelium; ve, vestibular epithelium; t, tongue; dm, dental mesenchyme.

PITX2 isoforms activate the *LEF-1* promoter. PITX2A and -C isoforms are expressed during dental morphogenesis. CHO cells transfected with the PITX2 isoforms revealed differences in their activations of the *LEF-1* promoter (Fig. 5A). PITX2A activated the *LEF-1* promoter at ~10-fold and PITX2C at ~18-fold (Fig. 5A). The active mutant β-catenin S37A construct and Lef-1 ΔN113 did not activate the *LEF-1* promoter, and cotransfection of both factors minimally activated *LEF-1* (Fig. 5A). This was expected, as the Lef-1 ΔN113 isoform does not contain the βBD. β-Catenin synergistically activated the *LEF-1* promoter in combination with PITX2A and -C (Fig. 5A). PITX2A and -C isoforms synergistically activated the *LEF-1* promoter in concert with Lef-1 ΔN113, indicating that the βBD is not required for this interaction with PITX2A and -C (Fig. 5A). Cotransfection of PITX2A and -C with Lef-1 ΔN113 and β-catenin resulted in a further synergistic activation of the *LEF-1* promoter (Fig. 5A).

The Lef-1 FL protein activated the *LEF-1* promoter at low levels, approximately fourfold, and at sevenfold in combination with β-catenin (Fig. 5B). β-Catenin synergistically activated the *LEF-1* promoter in combination with PITX2A and -C (Fig. 5B). The synergistic activation of the Lef-1 FL isoform with PITX2A and -C was similar to that of the Lef-1 ΔN113 isoform (Fig. 5B). Coexpression of PITX2 isoforms, Lef-1 FL, and β-catenin revealed increased synergistic activation (Fig. 5B). Transfected cells expressed the PITX2 isoforms, the Lef-1 ΔN113 and Lef-1 FL isoforms, and β-catenin (Fig. 5C, D, and E).

CHO cells were chosen, as they do not endogenously express *Pitx2* or the *Lef-1 FL* isoform and we could assay these activities independently of the response to endogenous activation. However, we repeated these experiments in the LS-8 tooth epithelial cell line, which endogenously expresses β-catenin, Lef-1, and Pitx2 isoforms (22). The relative differences in transactivation between the PITX2 isoforms in the presence of the various Lef-1 isoforms and/or β-catenin were similar to those of CHO cells (data not shown). These data support a β-catenin-independent Lef-1 interaction with PITX2 isoforms that regulates the *LEF-1* promoter and is not cell dependent.

Consistent with increased PITX2 transcriptional activation by β-catenin S37A (41), cells treated with LiCl increased PITX2 activation of the *LEF-1* promoter. LiCL is a potent glycogen synthase kinase 3 inhibitor and inhibits β-catenin phosphorylation and stabilizes the pool of cellular β-catenin similarly to Wnt signaling (36). The Lef-1 ΔN113 isoform was used to determine if endogenous β-catenin interaction with Lef-1 was required for the synergistic activation of the *LEF-1* promoter by PITX2. CHO cells were chosen for their low

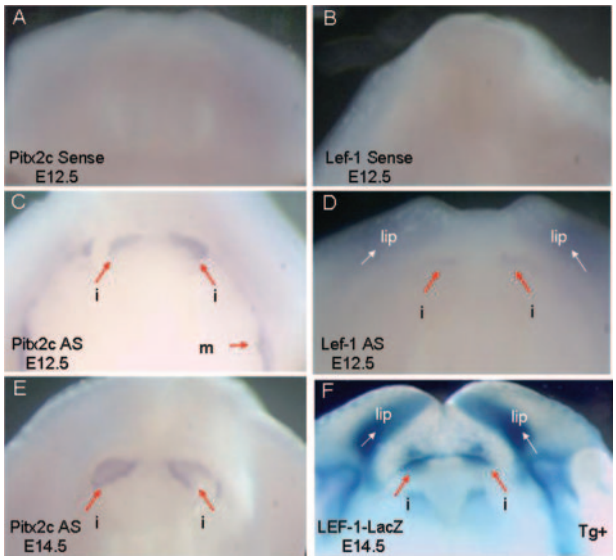

FIG. 3. Pitx2 and Lef-1 expression overlap in the developing tooth incisor. (A and B) Whole-mount in situ hybridization using Pitx2c sense probe (A) and Lef-1 sense probe (B) controls. (C and E) Whole-mount in situ hybridization using a Pitx2c antisense cRNA probe demonstrated *Pitx2c* mRNA expression in the mouse incisor (i) and molar (m) teeth at E12.5 (C) and E14.5 (E), respectively. Teeth are indicated by red arrows. (D) Lef-1 antisense in situ probe showing *Lef-1* expression in the mouse incisors (red arrows) and lip pad (lip, white arrows) at E12.5. (F) A 2.5-kb LEF-1–LacZ transgenic mouse showing LacZ staining of the mouse incisors (I, red arrows) and lip pad (lip) at E14.5 (white arrows).

levels of endogenous β-catenin and lack of Lef-1 FL or Pitx2 expression. LiCl treatment of CHO cells did not activate the *LEF-1* promoter in the presence of Lef-1 ΔN113; however, PITX2 transcriptional activation of the *LEF-1* promoter increased from 17-fold to 39-fold following LiCl treatment (data not shown). Cells cotransfected with PITX2 and Lef-1 ΔN113 and treated with LiCl revealed a dramatic increase in activation of the *LEF-1* promoter, from 28-fold to 95-fold (data not shown).

Mutation of the Lef-1 βBD demonstrates a β-catenin-independent pathway for PITX2 activation. The Lef-1 FL protein activity, in which the β-catenin interaction was abolished by specific mutations (Lef-1 M5), was compared to the Lef-1 ΔN113 N-terminally truncated protein. Lef-1 M5 contains six amino acid substitutions that disrupt the ability of the Lef-1 FL protein to interact with β-catenin (Fig. 6A) (28). Interestingly, this construct activated the *LEF-1* promoter ~17-fold; however, the synergistic responses with PITX2 were similar for both Lef-1 FL and Lef-1 M5 at ~55-fold (Fig. 6B). Cotransfection of β-catenin, PITX2, and Lef-1 M5 resulted in 100-fold activation of the *LEF-1* promoter (Fig. 6B). Thus, Lef-1 N-terminal residues do not affect its transcriptional activation with PITX2, nor is the activation dependent on β-catenin. A Lef-1 deletion construct (Lef-1ΔNΔβ-catenin) contains the Lef-1 HMG domain and the armadillo repeat and can act as a dominant active transcription factor (3). Lef-1ΔNΔβ-catenin activated the *LEF-1* promoter ~18-fold (Fig. 6B). Lef-1ΔNΔβ-

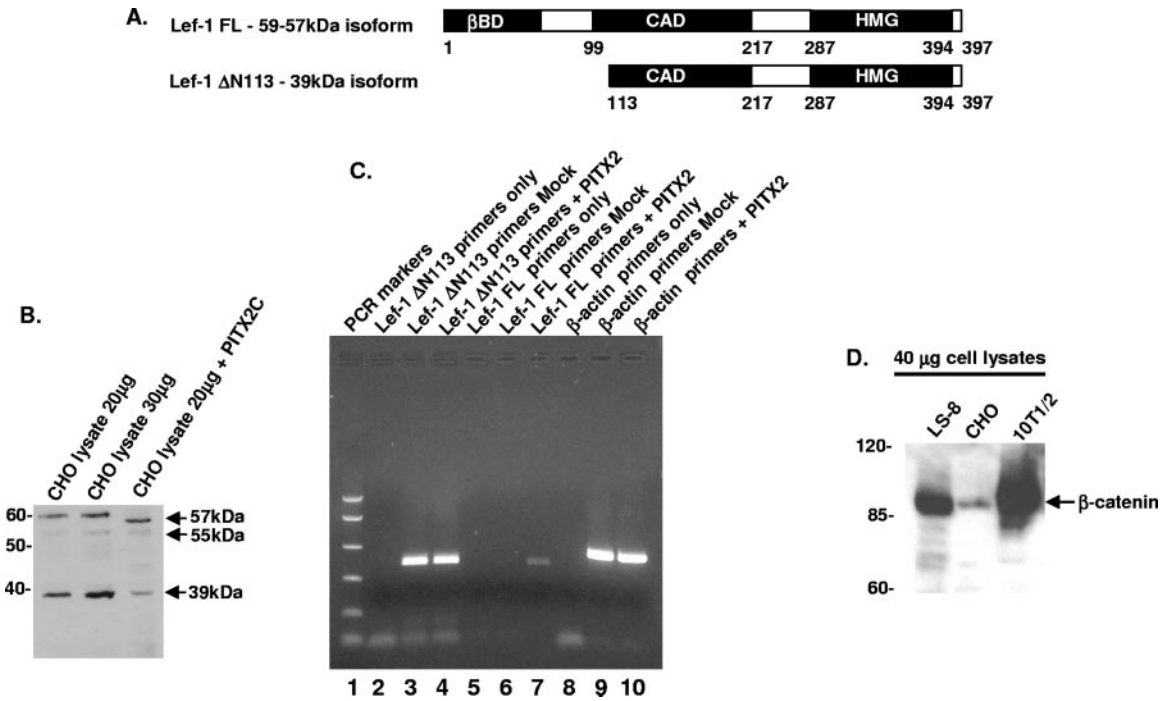

FIG. 4. PITX2 regulates endogenous *Lef-1* expression. (A) Schematic of two Lef-1 isoforms. The Lef-1 FL isoform is produced from the P1 promoter and encodes proteins of 57 and 55 kDa. The Lef-1 ΔN113 isoform is produced from the P2 promoter and encodes a protein of 39 kDa. The Lef-1 endogenously expressed isoforms also contain a CAD and an HMG domain. (B) PITX2-transfected CHO cells express the 57-kDa Lef-1 FL isoform. Molecular mass markers are shown on the left. Lef-1 protein was detected using the Lef-1 antibody. (C) RT-PCR assay using RNA isolated from either mock- or PITX2-transfected CHO cells. Mock- and PITX2-transfected CHO cells express the Lef-1 ΔN113 transcript, while only the PITX2-transfected cells express the Lef-1 FL transcript. β-Actin transcripts are equally expressed in both mock- and PITX2-transfected cells. (D) Expression of endogenous β-catenin in LS-8, C3H10T1/2, and CHO cell lysates. Approximately 40 μg of lysate was used in the β-catenin Western blot. Molecular weight markers are shown on the left.

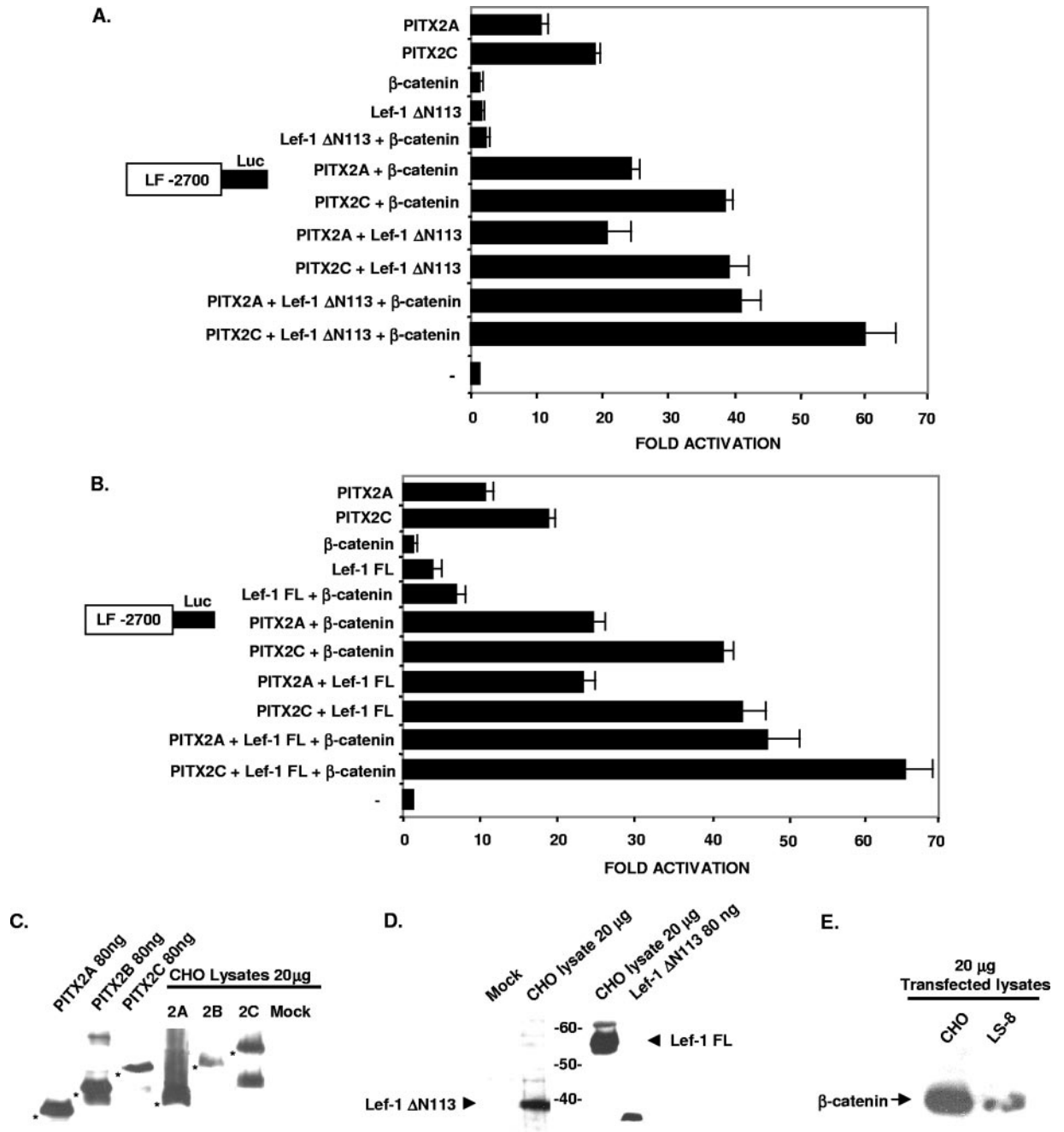

FIG. 5. PITX2 isoforms, β-catenin, and Lef-1 synergistically activate the *LEF-1* promoter in CHO cells. (A) CHO cells were transfected with the LEF-1 2700 luciferase reporter gene (5 μg). The cells were cotransfected with the CMV-PITX2A and -C isoforms and/or the CMV–Lef-1 ΔN113 short isoform and/or CMV–β-catenin S37A expression plasmids, or the CMV plasmid without PITX2, β-catenin, or Lef-1 ΔN113 (−) (2.5 μg). (B) CHO cells were transfected as in panel A, except the Lef-1 FL expression plasmid was used in the experiments. To control for transfection efficiency, all transfections included the SV-40 β-galactosidase reporter (0.5 μg). The cells were incubated for 24 h and then assayed for luciferase and β-galactosidase activities. The activities are shown as mean activation (*n*-fold) compared to the *LEF-1* promoter plasmid without PITX2, β-catenin, or Lef-1 expression and normalized to β-galactosidase activity (plus standard errors of the mean from five independent experiments for panel A and from four experiments for panel B). The mean *LEF-1* promoter luciferase activity with *PITX2* expression was about 250,000 light units per 15 μg protein, and the β-galactosidase activity was about 75,000 light units per 15 μg protein. (C) Western blot of transfected PITX2 isoforms in the CHO cell line using the PITX2 antibody. Whole-cell lysates from each cell line were prepared, and 20 μg of protein was run on a 10% SDS-polyacrylamide gel. The proteins were visualized using ECL reagents from Amersham. Pure PITX2 isoform proteins were used as a control at 80 ng. (D) Western blot of transfected Lef-1 FL and Lef-1 ΔN113 proteins from CHO cells using the same experimental procedure as for panel C. Purified Lef-1 ΔN113 protein was used as a control at 80 ng. The transfected Lef-1 ΔN113 protein migrated slightly more slowly than the pure protein due to the Myc-His tag on the transfected protein. The molecular weight markers are noted. (E) Transfected β-catenin in LS-8 and CHO cells; approximately 20 μg of lysate was used in the β-catenin Western blot.

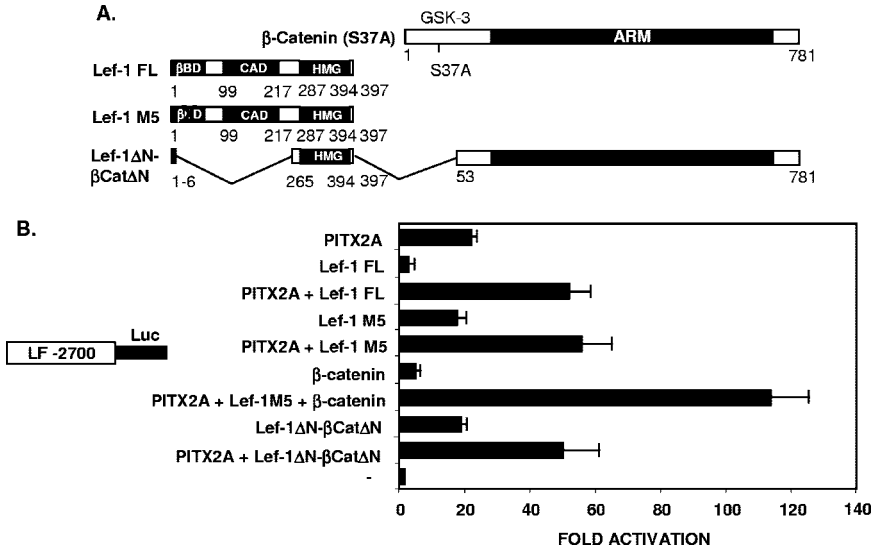

FIG. 6. β-Catenin induces *LEF-1* promoter activation by PITX2 independently of its interaction with Lef-1. (A) Schematic of β-catenin (S37A), Lef-1 FL, Lef-1 M5, and Lef-1ΔN-βCatΔN expression constructs. The M5 mutations in Lef-1 M5 are denoted by an X, which indicates the inability of this protein to interact with β-catenin. (B) CHO cells were transfected with the 2.5-kb LEF-1 luciferase reporter gene (5 μg). The cells were cotransfected with the CMV-PITX2A and/or CMV–Lef-1 FL, CMV–Lef-1 M5, and/or CMV–β-catenin; Lef-1ΔNΔβ-catenin expression plasmids; or the CMV plasmid without PITX2, β-catenin, or Lef-1 (−) (2.5 μg). To control for transfection efficiency, all transfections included the SV-40 β-galactosidase reporter (0.5 μg). The activities are shown as mean activation (*n*-fold) compared to the *LEF-1* promoter plasmid without PITX2, β-catenin, or Lef-1 expression and normalized to β-galactosidase activity (plus standard errors of the mean from five independent experiments).

catenin synergistically activated the *LEF-1* promoter in concert with PITX2, but not at the high levels observed when all three proteins were coexpressed (Fig. 6B). These data suggest that the Lef-1 context-dependent activation domain (CAD) is required for its synergistic activation with PITX2 and that the synergistic effect of Lef-1ΔNΔβ-catenin with PITX2 was due to the β-catenin protein.

The Lef-1 CAD is required for PITX2 transcriptional synergy. Deletion of the Lef-1 N terminus, including the CAD, but retaining the HMG domain (Lef-1 ΔN295) resulted in a loss of synergism with PITX2 in LS-8 cells (Fig. 7A and B). Further deletion of the HMG domain in construct Lef-1 ΔN363 also resulted in a loss of synergism with PITX2 (Fig. 7A and B). A C-terminal Lef-1 deletion removing only 34 C-terminal residues, Lef-1 ΔN113-ΔC34, did not synergistically activate the *LEF-1* promoter in combination with PITX2 (Fig. 7A and B). Interestingly, deletion of the Lef-1 HMG domain, but leaving the CAD domain intact (Lef-1 ΔN113-ΔC102), resulted in an increased synergism with PITX2 at ~24-fold (Fig. 7A and B). Because the Lef-1 HMG domain is required for DNA binding, the synergism of Lef-1 ΔN113-ΔC102 with PITX2 indicates that Lef-1 DNA binding is not necessary for this interaction. Furthermore, the Lef-1 HMG domain acts to repress the synergistic activation of the *LEF-1* promoter by PITX2. Similar results were observed using the PITX2C isoform and CHO cell transfections (data not shown). Nuclear expression of the truncated Lef-1 proteins in transfected cells has been reported (reference 21 and data not shown).

PITX2 and Lef-1 FL physically interact. Co-IP experiments detected interaction of the Lef-1 FL isoform with PITX2A in CHO cells (Fig. 8). The Lef-1 antibody immunoprecipitated the PITX2A–Lef-1 protein complex in transfected CHO cell lysates (Fig. 8, lane 4). In mock-transfected cell lysates, the Lef-1–PITX2A complex was not immunoprecipitated (Fig. 8, lane 1). Cell lysates were directly analyzed by Western blotting for PITX2 expression (Fig. 8, lanes 6 and 8). As a control, pure PITX2 protein (75 ng) was also loaded onto the gel (Fig. 8, lane 9). The transfected protein migrated slightly more slowly in the gel due to a Myc-His tag on the protein.

Lef-1 contains two PITX2 binding domains. To determine the locations of Lef-1 residues interacting with PITX2, GST–Lef-1 deletion proteins were isolated, immobilized, and incubated with purified PITX2C protein. Our GST pull-down experiments demonstrated that PITX2 can bind to the Lef-1 CAD and C-terminal flanking residues (Lef-1 ΔN113-ΔC102) to comprise PITX2 binding domain no. 1 (Fig. 9A and B). A second PITX2 binding domain (no. 2) is located in the C-terminal tail of Lef-1 and contains the last 34 residues (Lef-1 ΔN363) (Fig. 9A and B). A strong interaction occurred with the Lef-1 ΔN113-ΔC34 protein containing the distal HMG domain; however, in multiple experiments, PITX2 consistently bound the Lef-1 ΔN113-ΔC102 protein. PITX2C does not bind to the βBD (GST–Lef-1 βBD) located at the Lef-1 N terminus (Fig. 9B). The purified Lef-1 proteins used in the GST pull-down experiments are shown in the Coomassie blue-stained gel (Fig. 9C). We have previously reported that the C-terminal tail of PITX2 interacts with Lef-1 (41). All PITX2 isoforms contain identical C-terminal tails, and all can interact with Lef-1.

β-Catenin interacts with the PITX2 homeodomain. To determine the PITX2 β-catenin interaction site, GST-PITX2 and GST-PITX2 deletion proteins were immobilized on Sepharose beads and incubated with the β-catenin protein. β-Catenin bound to the PITX2 full-length protein, the N-terminally truncated PITX2 ΔN38 protein, and the C-terminally truncated PITX2 ΔC173 protein (Fig. 10A and B). However, it did not

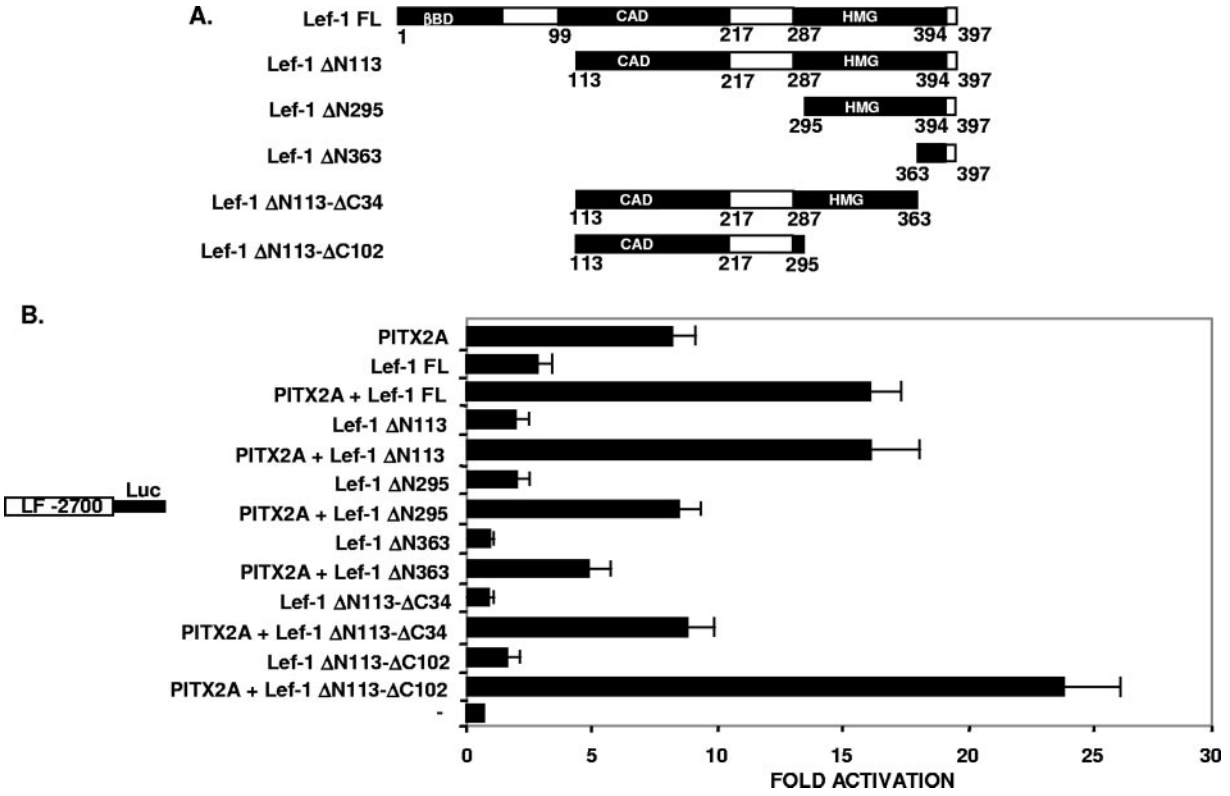

FIG. 7. The Lef-1 activation domain is required for synergism with PITX2. (A) Schematic of the Lef-1 deletion clones used to assay the synergistic response with PITX2. (B) LS-8 cells were transfected with the LEF-1 2700 luciferase reporter gene (5 μg). The cells were cotransfected with the CMV-PITX2A and/or CMV–Lef-1 FL, –Lef-1 ΔN113, and Lef-1 truncated expression plasmids or the CMV plasmid without PITX2 or Lef-1 (−) (2.5 μg). The transfections were performed as described in the legend to Fig. 5.

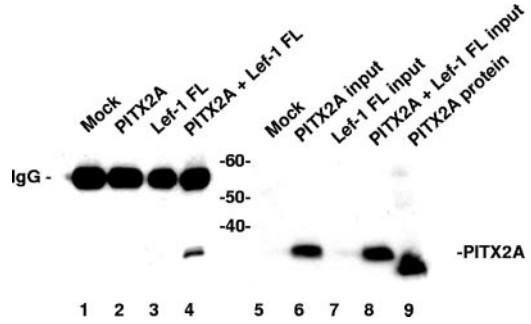

FIG. 8. Lef-1 and PITX2 physically interact. Lef-1 and/or PITX2A expression plasmids (2.5 μg) were transfected into CHO cells and incubated for 24 h. Cells were harvested and lysed, and the Lef-1–PITX2A protein complex was immunoprecipitated using the Lef-1 antibody. The immunoprecipitated complexes were resolved on a 10% polyacrylamide gel and transferred to a PVDF filter, and Western blotting was done using the PITX2 antibody. Lane 1 is the mock-transfected cell lysate IP, lane 2 is the PITX2-transfected IP, lane 3 is the Lef-1 FL-transfected IP, and lane 4 is the Lef-1 FL- and PITX2-cotransfected IP. Input controls are shown in lanes 5 to 8. As a molecular weight control, 75 ng of purified PITX2A protein was immunoblotted (lane 9). The transfected PITX2 migrated slightly more slowly due to the presence of a C-terminal Myc/His tag on the mammalian expression plasmids. Transfected Lef-1 is expressed in CHO cells, and a representative lysate is shown in Fig. 5E. Molecular weight markers are shown between lanes 4 and 5.

bind to the PITX2 C173 protein comprising the complete PITX2 C-terminal tail (Fig. 10A,B). Thus, β-catenin interacts with the homeodomain of PITX2 separately from the Lef-1 interaction domain in the PITX2 C-terminal tail.

β-Catenin and Lef-1 interact independently with PITX2. To demonstrate independent and simultaneous binding of both β-catenin and Lef-1 to PITX2, purified β-catenin and Lef-1 ΔN113 were incubated with immobilized GST-PITX2 homeodomain and GST-PITX2 C173 (Fig. 11A). Reactions were carried out with both β-catenin and Lef-1 ΔN113 in the binding reactions, and after processing, the reaction mixtures were split and one reaction mixture was probed with β-catenin antibody and the other with Lef-1 antibody. The Lef-1 ΔN113 protein was used, as it does not contain the βBD and does not interact with β-catenin. β-Catenin specifically interacted with the PITX2 homeodomain, and Lef-1 interacted with the PITX2 C terminus (Fig. 11B and C). Our results indicate that Lef-1 and β-catenin interact independently and simultaneously with the PITX2 protein through binding to separate protein interaction sites on the PITX2 protein. These results corroborate the transcriptional synergism observed when PITX2, Lef-1, and β-catenin are coexpressed.

DISCUSSION

Lef-1 expression is characterized by the production of several isoforms, and *Lef-1* transcripts arise through alternative

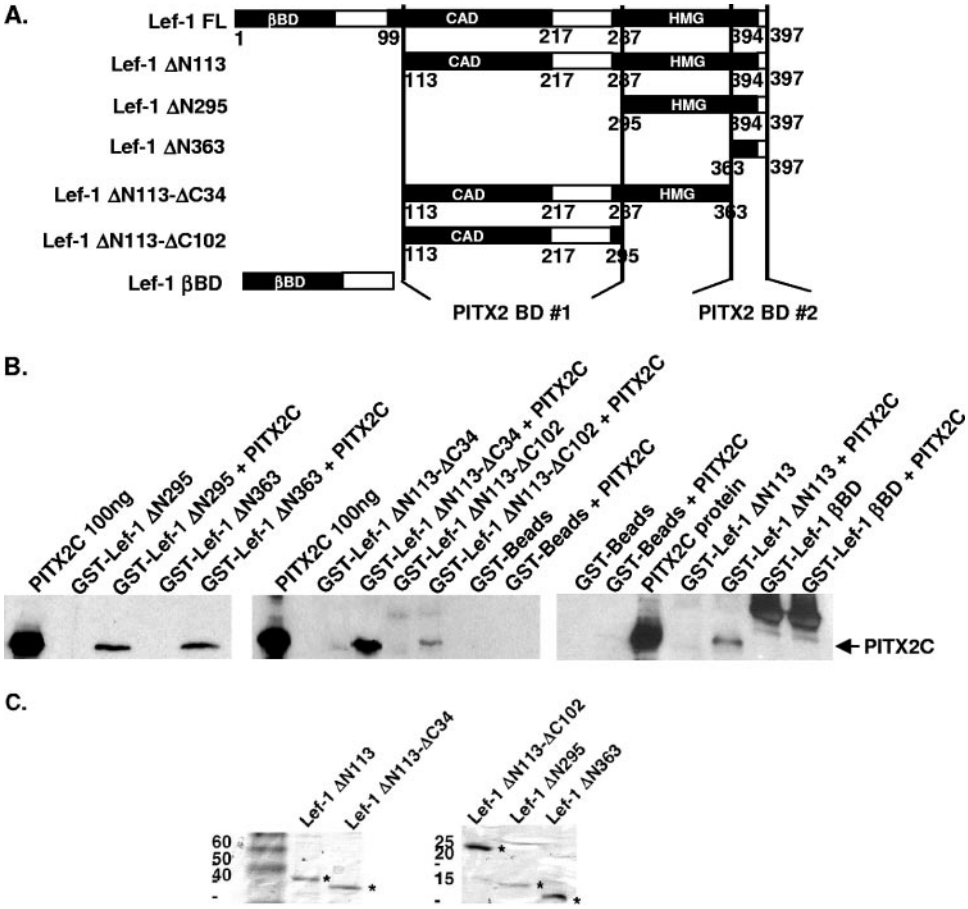

FIG. 9. PITX2 binds to two regions of the Lef-1 protein. (A) Schematic of the Lef-1 deletion proteins used in the GST pull-down assays. The locations of the two PITX2 interaction regions of Lef-1 are shown bracketed by lines and designated PITX2 binding domains (BD) 1 and 2. (B) GST–Lef-1 and truncated Lef-1 protein pull-down assay with bacterially expressed and purified PITX2C protein (100 ng). PITX2 binds to the Lef-1 ΔN295 protein, as well as the last 34 residues of the Lef-1 C-terminal tail (GST–Lef-1 ΔN363). This region is termed PITX2 BD 2. PITX2 binds to a region containing the CAD and 3′ flanking residues (Lef-1 ΔN113-ΔC102) and is termed PITX2 BD 1. As a control, GST beads were incubated with purified PITX2 to demonstrate the specificity of PITX2 binding to the GST–Lef-1 fusion proteins. PITX2 does not bind to the Lef-1 N-terminal βBD (GST–Lef-1 βBD). (C) Coomassie blue-stained gel of the purified Lef-1 proteins used in the binding assays. An asterisk denotes the proteins. Molecular weight markers are located on the left of the gels.

splicing and different promoters (8, 18, 26, 27, 34). The full-length *Lef-1* transcript contains a large 5′ untranslated region synthesized using a separate P1 promoter and utilizes a cap-independent mechanism for translation of the full-length Lef-1 containing the βBD (30, 34). This Lef-1 activity is countered by the expression of a truncated Lef-1 protein that lacks the βBD and can compete for binding to Wnt target genes. The shorter Lef-1 isoform is produced from a second (P2) promoter located in the second intron of the *LEF-1* locus (27, 30, 34). Recent studies have shown that the *LEF-1* promoter is transcriptionally responsive to Wnt/Tcf/β-catenin induction (12, 18, 34).

PITX2 and Lef-1 interact independently of β-catenin. PITX2A and -C isoforms synergistically activated the *LEF-1* promoter in concert with β-catenin. Because coexpression of the Lef-1 ΔN113 isoform with PITX2A and C isoforms and β-catenin resulted in further synergistic activation, these results suggested that Lef-1 interacts with PITX2 independently of β-catenin. Furthermore, LiCl treatment of transfected cells dramatically increased PITX2 transcriptional activation of the *LEF-1* promoter in the absence of Lef-1 (data not shown).

A Lef-1 interaction with PITX2 independent of β-catenin was further characterized using the Lef-1 M5 βBD mutant. β-Catenin expression resulted in a synergistic activation of the *LEF-1* promoter when cotransfected with PITX2 and Lef-1 M5. Thus, the βBD of Lef-1 is not required for its interaction with PITX2 or for the synergistic activation of PITX2 with β-catenin.

Induction of the *LEF-1* promoter in epithelial compartments during tooth organogenesis. *LEF-1* promoter expression in incisor teeth was limited to epithelial cells of the forming tooth bud and vestibular lamina in our studies using the 2.5-kb human *LEF-1* promoter. These findings suggest that regions contained within the 2.5-kb promoter allow proper regulation of gene expression in a subset of epithelial, but not mesenchymal, cells involved in tooth morphogenesis. The responsiveness of the 2.5-kb *LEF-1* promoter segment to Wnt signals in vitro is also consistent with *Wnt10b* expression at

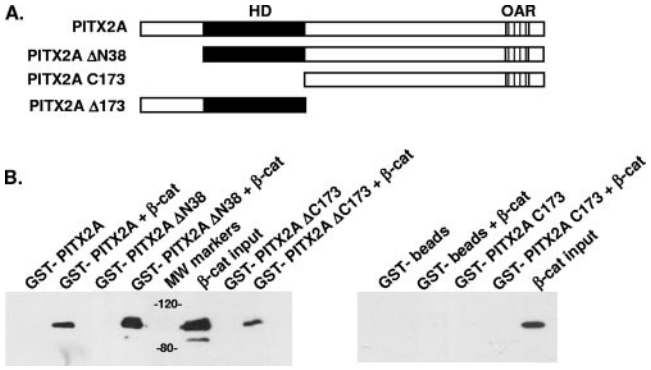

FIG. 10. β-Catenin interacts with the PITX2 homeodomain. (A) Schematic of the PITX2 deletion proteins used in the GST pull-down assays. HD, homeodomain; OAR, Otp and Aristaless domain. (B) GST-PITX2A and truncated PITX2A protein pull-down assay with bacterially expressed and purified β-catenin protein (50 ng). To demonstrate β-catenin binding to PITX2, β-catenin was incubated with several N-terminally and C-terminally truncated GST-PITX2 proteins. β-Catenin bound to the PITX2A, PITX2A ΔN38, and PITX2A ΔC173 proteins. β-Catenin did not bind to the PITX2A C173 C-terminal tail. As a control, GST beads were incubated with purified PITX2 to demonstrate the specificity of PITX2 binding to the GST–Lef-1 fusion proteins. β-Catenin binds to the PITX2 homeodomian.

early stages of tooth bud formation as an inductive signal for the *Lef-1* promoter (10).

PITX2 induces Lef-1 isoform-specific expression. The Lef-1 FL isoform (57 kDa) was specifically synthesized by the action of PITX2 in CHO cells. The Lef-1 FL isoform correlates with PITX2 activation of the Lef-1 57-kDa isoform from the P1 promoter (34). The increase in Lef-1 FL expression by PITX2 may result in a cell-proliferative effect. The Lef-1 FL isoform has been termed growth promoting because it is expressed during cell growth in undifferentiated, mitotically active cells. Furthermore, this Lef-1 isoform can interact with β-catenin and may stabilize nuclear β-catenin and allow increased Lef-1 and PITX2 transcriptional activity. Because PITX2 is required for early developmental events that are characterized by cell division, differentiation, and migration, the activation of the Lef-1 FL isoform could be essential for these functions. The shorter Lef-1 polypeptides could function as constitutive transcriptional repressors or competitive inhibitors of Wnt signaling by binding to Wnt-responsive elements in target genes. This would then disrupt β-catenin access and constitutively inhibit transcription by recruiting a repressor, and thus, it has been termed growth suppressing (6, 26, 34). However, PITX2 can interact with either Lef-1 isoform to synergistically activate transcription, which would inhibit the negative action by the Lef-1 short isoform. These interactions could play major roles during embryogenesis and in certain types of cancer.

PITX2 interacts with two domains of the Lef-1 protein. The Lef-1 amino terminus contains the βBD involved in Wnt signaling (4). The CAD (7, 21) and a region flanking the CAD are required for association with the Groucho corepressor and histone deacetylase (5). The C-terminal HMG domain is involved in DNA binding (20), and two regions within the HMG domain mediate the interaction with Smad factors (33). Two regions of Lef-1 were found to interact with Smad3 in the HMG domain. Our data suggest that two regions of Lef-1 interact with PITX2: the C-terminal region of the HMG domain containing 30 amino acids (Lef-1 ΔN363), which corresponds to the Smad3 MH1 binding domain, and a second region containing the CAD and flanking sequences (Lef-1 ΔN113-ΔC102). A previous report demon-

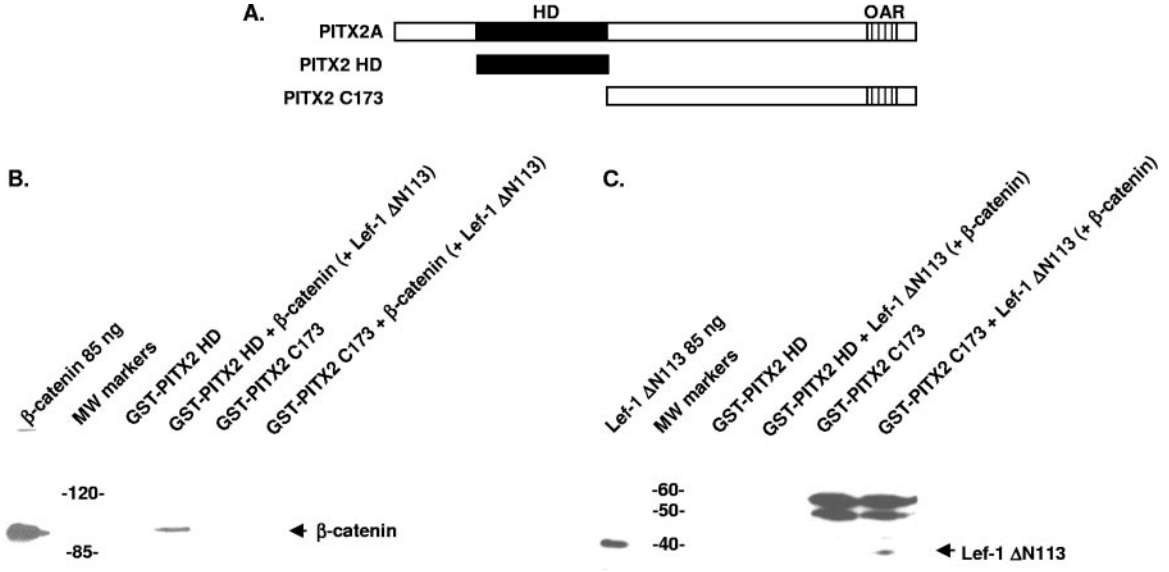

FIG. 11. Lef-1 and β-catenin bind independently and simultaneously to PITX2. (A) Schematic of the GST-PITX2 fusion proteins used in the GST pull-down assay. HD, homeodomain; OAR, Otp and Aristaless domain. (B) Lef-1 ΔN113 and β-catenin proteins (~85 ng) were incubated together with the immobilized GST-PITX2 constructs. β-Catenin bound to immobilized GST-PITX2 HD (homeodomain) but not to the GST-PITX2 C173 (C-terminal tail). (C) Lef-1 bound to immobilized GST-PITX2 C173 but not to the GST-PITX2 HD. Thus, β-catenin and Lef-1 bind simultaneously to different PITX2 residues.

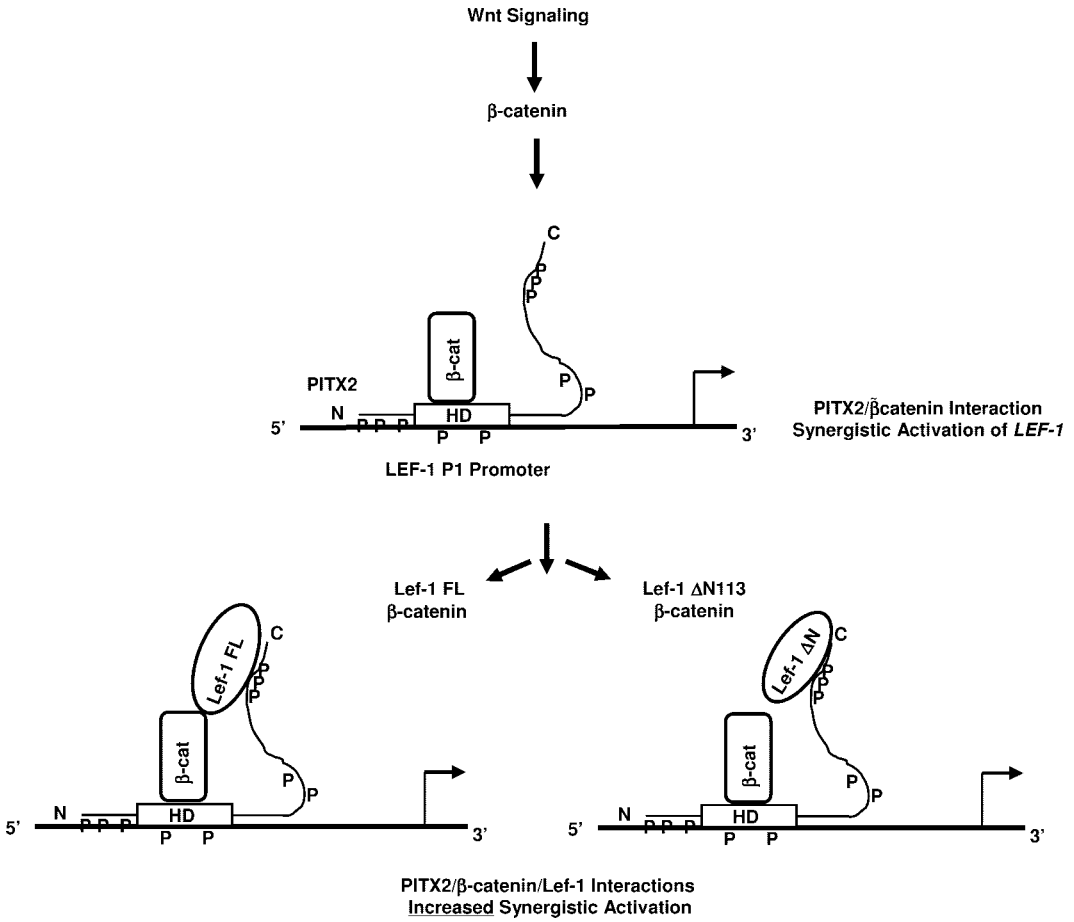

FIG. 12. Model for PITX2-independent binding of β-catenin and Lef-1 to increase PITX2 transcriptional activity. Depicted is PITX2 protein binding to DNA; Wnt signaling stabilizes β-catenin, which is translocated to the nucleus and interacts with PITX2 to synergistically activate the *LEF-1 P1* promoter. β-Catenin interacts with PITX2 through its homeodomain (HD). Nuclear β-catenin and Lef-1 FL interact with PITX2 and may also interact with each other through the Lef-1 βBD. Alternatively, β-catenin and Lef-1 ΔN113 interact with PITX2 but not with each other; however, both complexes yield increased synergistic activation of the *LEF-1* P1 promoter. Lef-1 interacts with PITX2 through the C-terminal tail. PITX2 interacts with Lef-1 through two sites C-terminal to the βBD and may allow Lef-1 and β-catenin to interact when complexed to PITX2. This complex allows PITX2 to be a highly active transcription factor in response to Wnt signaling and *Lef-1* expression. The 10 identified phosphorylation sites in the PITX2 protein are denoted by "P." It has been shown that phosphorylation of PITX2 facilitates DNA binding and protein interactions (16).

strated that Lef-1 FL can synergize with Smad4 in response to β-catenin activation to activate the *Msx2* promoter (29). Furthermore, Dlx2 can interact with Lef-1 through these sites, which increases the transcriptional activity of Dlx2, similar to PITX2 (11). Competition between factors for these sites could add to the complexity of Lef-1 transcriptional activity.

Wnt signaling and β-catenin regulate PITX2 transcriptional activity. β-Catenin is required for normal tooth development and is expressed in the dental epithelium in an overlapping fashion with *Pitx2* and *Lef-1* (17). Our data demonstrate a mechanism for Wnt signaling in the transcriptional activity of PITX2. We speculate that β-catenin displaces an inhibitory factor or complex that allows PITX2 to become active. A Gal4-Pitx2 fusion protein demonstrated repressor activity, and it was proposed that β-catenin acts in Pitx2 derepression (31).

β-Catenin interacts with the homeodomain of PITX2, and Lef-1 interacts with the PITX2 C-terminal tail. These separate interactions have a combinatorial/synergistic effect on the transcriptional properties of PITX2. The Lef-1 FL or the Lef-1 ΔN113 isoform can interact with PITX2 independently of β-catenin, and β-catenin can interact with PITX2 independently of Lef-1. We propose a model in which β-catenin can interact with PITX2, and depending on the Lef-1 isoform interacting with PITX2, β-catenin may be able to directly interact with the Lef-1 FL isoform while complexed with PITX2 to activate gene expression (Fig. 12). Therefore, β-catenin interacting with the PITX2 homeodomain or Lef-1 interacting with the PITX2 C-terminal tail would independently increase PITX2 transcriptional activity. However, when both β-catenin and either Lef-1 isoform are present, they would form a potent PITX2 transcriptional complex (Fig. 12). The activation of *Lef-1* expression would then provide a feedback mechanism for continued Lef-1 interaction with PITX2 to continuously drive *Lef-1 FL* expression.

Interestingly, a recent report has demonstrated a role for

Lef-1 in the survival of dental epithelial cells during tooth development (40). In *Lef-1*-null mutant mice, the dental epithelium underwent increased apoptosis and subtle changes in cell proliferation. In *Pitx2*-null mutant mice, tooth development is arrested at an earlier stage than in *Lef-1* mutants, and *Pitx2* mutant mice have cell proliferation and migration defects associated with defective tooth morphogenesis (37). Thus, the differential activation of Lef-1 isoforms may play a role in the ability of PITX2 to regulate cell migration and/or proliferation. PITX2 activation of the *LEF-1* promoter correlates with the temporal and spatial expression patterns of these two factors in the dental epithelium.

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